

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
8 January 2004 (08.01.2004)

PCT

(10) International Publication Number
WO 2004/003817 A1

(51) International Patent Classification⁷: **G06F 19/00**,
C12Q 1/70, C12N 9/50

(21) International Application Number:
PCT/EP2003/050277

(22) International Filing Date: 30 June 2003 (30.06.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/392,753 1 July 2002 (01.07.2002) US

(71) Applicant (for all designated States except US): **TI-
BOTEC PHARMACEUTICALS LTD.** [IE/IE]; Unit 4,
Block 4B, Blanchardstown Corporate Park, Blanchard-
stown, 15 Dublin (IE).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **DE MEYER,**
Sandra [BE/BE]; Sijssesstraat 22, B-2340 Beerse (BE).
AZIJN, Hilde [BE/BE]; Wijngaardlaan 8, B-3001 Leuven
(BE). **DE BETHUNE, Marie-Pierre T.M.M.G** [BE/BE];
Twee Leeuwenstraat 15, B-3078 Everberg (BE).

(74) Agent: **DAELEMANS, Frank**; Tibotec-Virco Comm.
VA, Generaal De Wittelaan L11 B3, B-2800 Mechelen
(BE).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,
MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC,
SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA,
UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),
Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE,
ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO,
SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM,
GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declaration under Rule 4.17:

— of inventorship (Rule 4.17(iv)) for US only

Published:

— with international search report
— before the expiration of the time limit for amending the
claims and to be republished in the event of receipt of
amendments

For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

(54) Title: MUTATIONAL PROFILES IN HIV-1 PROTEASE CORRELATED WITH PHENOTYPIC DRUG RESISTANCE

(57) Abstract: The present invention is directed to the field of nucleic acid diagnostics and the identification of base variation in target nucleic acid sequences. More particularly, the present invention relates to the use of such genotypic characterization of a target population of HIV and the subsequent association, i.e., correlation, of this information to phenotypic interpretation in order to correlate virus mutational profiles with drug resistance. The invention also relates to methods of utilizing the mutational profiles of the invention in drug development, i.e., drug discovery, drug design, drug modification, and therapy, treatment design, clinical management and diagnostic analysis.

WO 2004/003817 A1

PCT/EP2003/050277 22 DEC 2004

**MUTATIONAL PROFILES IN HIV-1 PROTEASE CORRELATED WITH
PHENOTYPIC DRUG RESISTANCE**

5 The present invention is directed to the field of nucleic acid diagnostics and the identification of base variation in target nucleic acid sequences. The invention provides novel mutations or mutational profiles of HIV-1 protease gene correlated with a phenotype causing alterations in sensitivity to anti-HIV drugs. The present invention also relates to the use of genotypic characterization of a target population of HIV and the subsequent association, i.e. correlation, of this information to phenotypic
10 interpretation in order to correlate virus mutational profiles with drug resistance. The invention further relates to methods of utilizing the mutational profiles of the invention in databases, drug development, i.e., drug design, and drug modification, therapy and treatment design and clinical management.

The development and standardization of plasma HIV-1 RNA quantification
15 assays has led to the use of viral load measurements as a key therapy response monitoring tool. The goal of antiretroviral therapy is to reduce plasma viremia to below the limit of detection on a long-term basis. However, in a significant number of patients, maximal suppression of virus replication is not achieved and for those in whom this goal is reached, a significant number experience viral load rebound. Viral load data provide
20 no information on the cause of the failure.

Therapy failure may be due to a number of factors, including insufficient antiviral activity of the regimen, individual variations in drug metabolism and pharmacodynamics, difficulties in adhering to dosing regimen, requirements for treatment interruption due to toxicity, and viral drug resistance. Moreover, drug resistance may develop in a patient
25 treated with sub-optimal antiretroviral therapy or a patient may be infected with drug-resistant HIV-1. Although drug resistance may not be the primary reason for therapy failure, in many cases any situation which permits viral replication in the presence of an inhibitor sets the stage for selection of resistant variants.

Viral drug resistance can be defined as any change in the virus that improves replication
30 in the presence of an inhibitor. HIV-1 drug resistance was first described in 1989 and involved patients that had been treated with zidovudine monotherapy (Larder, B.A., et al., Science 243, 1731-1734 (1989)). Emergence of resistance is almost always being observed during the course of treatment of patients with single antiretroviral drugs. Similarly, *in vitro* passage of viral cultures through several rounds of replication in the
35 presence of antiretroviral compounds leads to the selection of viruses whose replication cycle is no longer susceptible to the antiretroviral compounds used. Resistance development has also been observed with the introduction of dual nucleoside reverse transcriptase inhibitors (NRTI) combination therapy as well as during the administering

of the more potent non- nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs) and combinations thereof. Individual antiretroviral agents differ in the rate at which resistance develops: selection for resistant variants may occur within weeks of treatment or resistance may emerge after a longer treatment period.

5 Extensive genetic analysis of resistant viral isolates generated through *in vivo* or *in vitro* selection has revealed that resistance is generally caused by mutations at some specific site(s) of the viral genome. The mutational patterns that have been observed and reported for HIV-1 and that are correlated with drug resistance are very diverse: some antiretroviral agents require only one single genetic change, while others require
10 multiple mutations for resistance to appear. A summary of mutations in the HIV genome correlated with drug resistance has been compiled (See e.g. Schinazi, Int. Antiviral News. 6, 65 (2000)). Electronic listings with mutations are available at different web locations such as hiv-web.lanl.gov/content/index, www.hivb.stanford.edu, and www.hivresistanceweb.com.

15 A genetic mutation is normally written in reference to the wild type virus, *i.e.*, K101N refers to replacement of a Lysine at codon 101 with a Asparagine (The Molecular Biology of the Cell, 1994, Garland Publishing, NY). However, the mutations of the invention do not depend on the wild-type example listed in order to be within the practice of the invention. For example, the mutation 101N, refers to an Asparagine at
20 the 101 codon regardless of the whether there was a Lysine at 101 prior to mutation. Alternatively, it may be said that a particular amino acid occurs at a given position, wherein "position" is equivalent to "codon". Mutations can also be identified in nucleic acids such as RNA, DNA, mRNA.

The degree of susceptibility of a genetic variant to an antiretroviral compound is
25 expressed herein relative to the wild-type virus (HIV IIIB/LAI reference sequence) as found, for example, in GenBank, the sequence of which is hereby incorporated by reference (K03455, gi 327742, M38432). An alteration in viral drug sensitivity is defined as a change in resistance or a change in susceptibility of a viral strain to said drug. Susceptibilities are generally expressed as ratios of EC₅₀ or EC₉₀ values (the EC₅₀
30 or EC₉₀ value being the drug concentration at which 50% or 90% respectively of the viral population is inhibited from replicating) of a viral strain under investigation compared to the wild type strain. Hence, the susceptibility of a viral strain can be expressed as a fold change in susceptibility, wherein the fold change is derived from the ratio of for instance the EC₅₀ values of a mutant viral strain compared to the wild type.
35 In particular, the susceptibility of a viral strain or population may also be expressed as resistance of a viral strain, wherein the result is indicated as a fold increase in EC₅₀ as compared to wild type EC₅₀.

As antiretroviral drugs are administered for longer periods, mostly in combination with each other, and as new antiretrovirals are being developed and added to the present drugs, new resistance-correlated genetic variants are being identified. Of particular importance is that the combination of antiretroviral agents can influence resistance characteristics.

Once viral resistance has developed, salvage therapy options may be severely restricted due to cross-resistance within each drug class. This is as important for initial treatment as for when a therapy change is called for in order to minimize the emergence of resistance and improve the long-term prognosis of the patient. The choice of therapy regimen will be supported by knowledge of the resistance profile of the circulating virus population. Additionally, therapy combinations will have a greater chance of being effective if they include agents that have a demonstrated potential of suppressing a particular virus population.

A number of applications describe the occurrence of mutations in HIV and their correlation to the development of drug resistance (WO 00/73511; WO 02/33402; WO 02/22076; WO 00/78996). The instant invention adds to the art mutations in the protease gene and their correlation i.e. association to viral drug resistance.

DETAILED DESCRIPTION OF THE INVENTION

The knowledge that mutations at position 41 and 70 correlate with a fold change in resistance can be applied in certain useful methods. The present invention relates to methods for evaluating the effectiveness of a protease inhibitor, based on the presence of at least one mutation selected from 41S, 41T, 41I, 41K, 41G and 70E, in HIV protease.

In particular, the present invention relates to methods for evaluating the effectiveness of a protease inhibitor, based on the presence of at least one mutation selected from 41T, 41I, 41K, 41G and 70E, in HIV protease. The presence of at least one of said mutations correlates to a fold change in susceptibility or resistance of an HIV viral strain towards at least one protease drug. The effectiveness of a protease inhibitor in the presence of at least one of said mutations may be determined using e.g. enzymatic, phenotypic and genotypic methods. The correlation between the mutational profiles in HIV protease and drug usage may be useful for clinical toxicological and forensic applications. A combined approach involving genotypic and phenotypic resistance testing to correlate mutations with resistance phenotypes may be used. More in particular, the present invention provides a correlation between at least one strain of HIV having at least one mutation in HIV protease selected from 41T and 70E and a fold change in resistance. In one aspect of the invention, the HIV protease mutations, 41T and 70E, are both present in a viral strain.

The effectiveness of a protease inhibitor as an antiviral therapy for a patient infected with at least one HIV strain comprising mutant protease may be determined using a method comprising: (i) collecting a sample from an HIV-infected patient; (ii) determining whether the sample comprises a HIV protease having at least one mutation selected from 41S, 41T, 41I, 41K, 41G, and 70E; and (iii) correlating the presence of said at least one mutation of step (ii) to a change in effectiveness of said protease inhibitor. In particular, the effectiveness of a protease inhibitor as an antiviral therapy for a patient infected with at least one HIV strain comprising mutant protease may be determined using a method comprising: (i) collecting a sample from an HIV-infected patient; (ii) determining whether the sample comprises a HIV protease having at least one mutation selected from 41T, 41I, 41K, 41G, and 70E; and (iii) correlating the presence of said at least one mutation of step (ii) to a change in effectiveness of said protease inhibitor.

In general a change in effectiveness can be expressed as a fold change in resistance. The fold change may be determined using a cellular assay including a cytopathogenic assay or the Antivirogram® (WO 97/27480). Alternatively, the fold change in susceptibility may be derived from database analysis such as the VirtualPhenotype™ (WO 01/79540). A decrease in susceptibility vis-à-vis the wild type virus correlates to an increased viral drug resistance, and hence reduced effectiveness of said drug. To determine the viral drug susceptibility the activity of the mutant enzyme may be compared to the activity of a wild type enzyme. In phenotyping assays pseudotyped viruses may be used. The mutations present in HIV protease may be determined at the nucleic acid or amino acid level using sequencing or hybridization techniques. A report may be generated that shows the region of the patient virus that has been sequenced, including at least one mutation selected from 41S, 41T, 41I, 41K, 41G and 70E, in particular, including at least one mutation selected from 41T, 41I, 41K, 41G and 70E. The report may include antiretroviral drugs, drug(s) for which a known resistance-associated mutation has been identified and/or to what extent the observed mutations selected from at least 41S, 41T, 41I, 41K, 41G and 70E are indicative of resistance to said drugs. In particular, the report may include drug(s) for which a known resistance-associated mutation has been identified and/or to what extent the observed mutations selected from at least 41T, 41I, 41K, 41G and 70E are indicative of resistance to said drugs. HIV may be present in combinations of several strains. This may result in the presence of multiple mutations at a particular amino acid, including partial mutations. Partial mutations include the combination of the wild amino acid and a mutant amino acid at a particular position. Examples thereof include partial mutations at position 41 in HIV protease, including 41R/S, 41S/R, 41R/K, 41G/R, in particular 41R/K, 41G/R. The sample to be evaluated

can be a bodily fluid including blood, serum, plasma, saliva, urine, or a tissue including gut tissues.

5 The fact that particular data correlate, indicates that a causal relationship exists between the data. Hence, a particular result renders a particular conclusion more likely than other conclusions.

10 A drug effective against mutant HIV protease may be identified by a method, comprising: (i) providing a nucleic acid comprising HIV protease comprising at least one mutation chosen from 41S, 41T, 41I, 41K, 41G and 70E; (ii) determining a phenotypic response to said drug for said HIV recombinant virus; and (iii) identifying a drug effective against mutant HIV based on the phenotypic response of step (ii). In particular, a drug effective against mutant HIV protease may be identified by a method, comprising: (i) providing a nucleic acid comprising HIV protease comprising at least
15 one mutation chosen from 41T, 41I, 41K, 41G and 70E; (ii) determining a phenotypic response to said drug for said HIV recombinant virus; and (iii) identifying a drug effective against mutant HIV based on the phenotypic response of step (ii). The nucleic acid comprising HIV of step (i) may be recombined into a proviral nucleic acid deleted for said sequence to generate a recombinant HIV virus.

20

Identifying a drug is defined as making a selection of drugs clinically available based on the effectiveness of said drug. In addition to the selection of clinically available drugs, identifying also relates to the selection of clinical drug candidates. The phenotypic response may be determined using cellular assays such as the Antivirogram®. An
25 effective drug against mutant HIV comprising at least one mutation in protease selected from 41T and 70E, is defined as a drug having a phenotypic response expressed, as e.g. a fold change in susceptibility lower than a defined cut-off that may be determined for a drug.

30 An other useful method for identifying a drug effective against mutant HIV protease comprising: (i) providing a HIV protease comprising at least one mutation chosen from 41S, 41T, 41I, 41K, 41G and 70E;

- (ii) determining the activity of said drug on said HIV protease;
- (iii) determining the activity of said drug on wild type HIV protease;
- 35 (iv) determining the ratio of the activity determined in step (iii) over the activity determined in step (ii);
- (v) identifying an effective drug against mutant HIV based on the ratio of step (iv).

In particular, a useful method for identifying a drug effective against mutant HIV protease comprising: (i) providing a HIV protease comprising at least one mutation chosen from 41T, 41I, 41K, 41G and 70E;

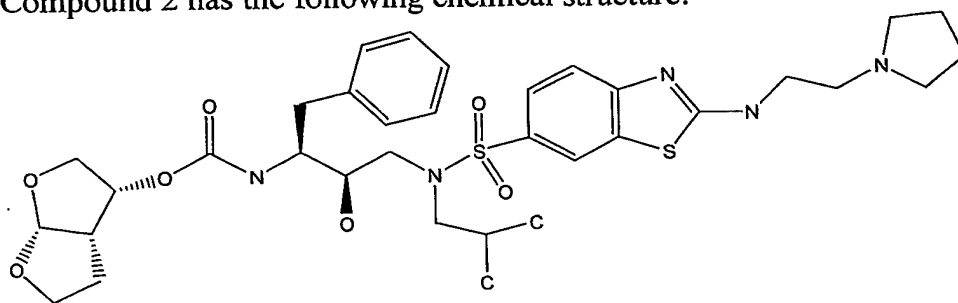
- (ii) determining the activity of said drug on said HIV protease;
- (iii) determining the activity of said drug on wild type HIV protease;
- (iv) determining the ratio of the activity determined in step (iii) over the activity determined in step (ii);
- (v) identifying an effective drug against mutant HIV based on the ratio of step (iv).

A ratio lower than a defined cut-off value that can be specific for said drug is indicative that the drug is effective against mutant HIV (WO 02/33402).

The activity of said drug on said HIV protease, having at least one mutation selected from 41S, 41T, 41I, 41K, 41G and 70E, in particular 41T, 41I, 41K, 41G and 70E, can be determined in an enzymatic assay, wherein the mutant protease, is compared to the wild type enzyme by its enzymatic characteristics (e.g. Maximal velocity (V_{max}), Michaelis-Menten constant (K_m), catalytic constant (k_{cat})). An activity means any output generated by the assay including fluorescence, fluorescence polarization, luminiscence, absorbance, radioactivity, resonance energy transfer mechanisms, magnetism. The use of fluorescent substrates to measure the HIV protease activity was described by e.g. Matayoshi et al. [Science 1990, 247, 954], Tyagi et al. [Anal. Biochem. 1992, 200(1), 143], Toth et al. [Int. J. Pept. Protein Res. 1990, 36(6), 544] and Wang et al. [Tetrahedron 1990, 31(45), 6493] and in several patent applications [see e.g. WO99/67417; EP428000, EP518557]. A suitable substrate for the enzymatic determination is R-E(EDANS)-S-Q-N-Y-P-I-V-Q-K(DABCYL)-R-OH (Science, 1989, 247, 954-958). Alternatively HPLC based methods may be used to determine the activity.

The response of a mutant HIV protease having at least one mutation selected from 41S, 41T, 41I, 41K, 41G and 70E, in particular 41T, 41I, 41K, 41G and 70E, may be expressed as viral fitness (WO 00/78994). This viral fitness can be defined as the ability of a viral strain to replicate in the presence or absence of a component, such as a protease inhibitor. This viral fitness is dependent on a combination of factors including viral factors which include mutations occurring in viral proteins, such as the mutations described herein, host factors which include immune responses, differential expression of membrane proteins and selective pressures which include the presence of antiviral agents such as protease inhibitors.

Interestingly, protease inhibitors that can be used in the present methods include Nelfinavir, Saquinavir, Indinavir, Amprenavir, Tipranavir, Lopinavir, Ritonavir, Palinavir, Atazanavir, Mozenavir, Fosamprenavir, compound 1 (Carbamic acid, [(1S,2R)-3-[[[(4-aminophenyl)sulfonyl](2-methylpropyl)amino]-2-hydroxy-1-(phenylmethyl)propyl]-, (3R,3aS,6aR)-hexahydrofuro[2,3-b]furan-3-yl ester, compound 1), and compound 2, which has been described as a HIV protease inhibitor in WO02/083657 and which can be prepared according to the procedures described therein. Compound 2 has the following chemical structure:



In an embodiment, the protease inhibitor is selected from Indinavir, Saquinavir, Lopinavir, Nelfinavir, compound 1, and compound 2. In particular, the protease inhibitor is selected from Indinavir, Saquinavir, Lopinavir and compound 1.

Conveniently, the methods of the present invention are performed using samples of an HIV-infected patient that has been treated with at least a protease inhibitor. More in particular, the patient contains mutant viruses bearing at least one additional mutation at position in the HIV protease selected from 10, 30, 33, 46, 47, 50, 54, 63, 71, 74, 77, 82, 84, 88 or 90. Even more in particular, the mutant viruses are resistant towards the therapy the patient is taken.

A vector comprising an HIV sequence having at least one mutation in the HIV protease gene chosen from 41S, 41T, 41I, 41K, 41G and 70E may be useful for the phenotypic analysis. In particular, a vector comprising an HIV sequence having at least one mutation in the HIV protease gene chosen from 41T, 41I, 41K, 41G and 70E may be useful for the phenotypic analysis. The present knowledge about the correlation between a fold change in susceptibility and the presence of at least one mutation selected from 41S, 41T, 41I, 41K, 41G and 70E in HIV protease can be used to prepare an isolated and purified HIV protease sequence having at least one mutation selected from 41S, 41T, 41I, 41K, 41G and 70E. In particular, the knowledge about the correlation between a fold change in susceptibility and the presence of at least one mutation selected from 41T, 41I, 41K, 41G and 70E in HIV protease can be used to prepare an isolated and purified HIV protease sequence having at least one mutation selected from 41T,

41I, 41K, 41G and 70E.

The knowledge of the mutations of the present invention offers the possibility to develop probes and primers directed to said mutations. An isolated and purified oligonucleotide comprising a HIV protease sequence of 5 to 100 bases comprising at least one mutation chosen from 41S, 41T, 41I, 41K, 41G and 70E, may be useful for in vitro diagnosis of viral drug resistance. In particular, an isolated and purified oligonucleotide comprising a HIV protease sequence of 5 to 100 bases comprising at least one mutation chosen from 41T, 41I, 41K, 41G and 70E, may be useful for in vitro diagnosis of viral drug resistance. Suitable oligonucleotides for nucleic acid amplifying technologies contain 5 to 35 nucleic acid bases. Suitably such oligonucleotides contain 15 to 30 nucleic acid bases. An oligonucleotide may contain the mutant base at the 3' end so as to enable the detection of the mutant using PCR. Oligonucleotides may also be used as probes including molecular beacons (Tyagi, Nature Biotechnol 1998, 16(1) 49-53), and TaqMan probes.

A computer system comprising at least one database correlating the presence of at least one mutation in a human immunodeficiency virus protease and fold change in susceptibility of at least one strain of HIV to a protease inhibitor, comprising at least one record corresponding to a correlation between at least one mutation selected from 41S, 41T, 41I, 41K, 41G and 70E, in particular 41T, 41I, 41K, 41G and 70E, and treatment with at least a protease inhibitor can be used for evaluating resistance towards therapy.

A neural network that predicts the development of therapeutic agent resistance or sensitivity against at least one viral strain comprising at least one mutation selected from 41S, 41T, 41I, 41K, 41G and 70E can be used (WO 01/95230). In particular, a neural network that predicts the development of therapeutic agent resistance or sensitivity against at least one viral strain comprising at least one mutation selected from 41T, 41I, 41K, 41G and 70E can be used (WO 01/95230).

Genotyping methodologies

Resistance of HIV to antiretroviral drugs may be determined at the genotypic level by identifying mutations in the HIV-1 genome and by inferring the resistance of HIV-1 to antiretroviral drugs through searching for mutational patterns known to correlate with resistance. Assays for detection of mutations in HIV-1 may be based on polymerase chain reaction (PCR) amplification of viral genomic sequences. These amplified sequences are then analyzed using either hybridization or sequencing techniques. Hybridization-based assays include primer-specific PCR, which makes use of synthetic

oligonucleotides designed to allow selective priming of DNA synthesis. See Larder, B.A., et al., AIDS 5, 137-144 (1991); Richman, D.D., et al., J. Infect. Dis. 164, 1075-1081 (1991); Gingeras, T.R., et al., J. Infect. Dis. 164, 1066-1074 (1991). Only when primer sequences match the target sequence (wild-type or mutant) at the 3' end, is amplification of target sequences possible and DNA fragments are produced.

Knowledge of the primer sequences allows one to infer the sequence of the viral isolate under investigation, but only for the region covered by the primer sequences. Other hybridization-based assays include differential hybridization (Eastman, P.S., et al., J. Acq. Imm. Def. Syndr. Human Retrovirol. 9, 264-273 (1995); Holodniy, M., et al., J. Virol. 69, 3510-3516 (1995); Eastman, P.S., et al., J. Clin. Micro. 33, 2777-2780 (1995).); Line Probe Assay (LiPA[®] HIV-11 RT, Innogenetics) (Stuyver, L., et al., Antimicrob. Agents Chemotherap. 41, 284-291 (1997)); and biochip technology such as GENECHIP[®] technology (Affymetrix) (D'Aquila, R.T. Clin. Diagnost. Virol. 3, 299-316 (1995); Fodor, S.P.A. et al., Nature 364, 555-556 (1993); Fodor, S.P.A. Nature 227, 393-395 (1997). The sequence may also be determined using mass spectroscopic technologies. DNA sequencing assays provide information on all nucleotides of the sequenced region. Sequencing results may be reported as amino acid changes at positions in the protease gene and the reverse transcriptase gene compared to the wild-type reference sequence. The changes included in the genotyping report may be limited to mutations at positions known to manifest drug resistance-associated polymorphisms. Polymorphisms at positions not associated with drug resistance may be omitted.

Phenotyping methodologies

Phenotyping assays measure the ability of a replicating virus to grow in the presence of compounds compared to a wild-type reference virus such as e.g. HIV-1/LAI, HIV-1/NL4.3, HIV-1/HXB2 or e.g. HIV-2/ROD. Alternatively, phenotyping assays are performed with pseudotyped viruses not able to replicate (WO 02/38792). Consequently, these assays directly measure the degree of viral susceptibility to specific inhibitors. In this case, one measures the effect of all mutational interactions, the effects of genetic changes as yet unknown or not previously identified, the effect of the background genotype, etc., on the phenotype. Some phenotypic assays are discussed below.

Cytopathic effect assay (CPE assay)

Determination of the antiviral activity of a compound was done as described in Pauwels R. *et al.* (J Virol Methods 1988; 20(4):309-21). Various concentrations of the test compounds were brought into each well of a flat-bottom microtiter plate. Subsequently, HIV and MT4 cells were added to a final concentration of 200-250 50% cell culture infectious doses (CCID₅₀)/well and 30,000 cells/well, respectively. After 5 days of incubation (37°C, 5% CO₂), the cytopathic effect of the replicating virus was determined

by the tetrazolium colorimetric MTT method. The dose protecting 50% of the cells from virus cytopathic effect was defined as the EC₅₀, while the dose achieving 90% protection was defined as the EC₉₀.

Reporter gene assay

- 5 The reporter gene assay used MT4-LTR-EGFP cells. Upon infection by HIV-1, the expression of the viral tat product increases transcription from the HIV-1 LTR promoter, leading to high-level expression of the reporter gene product. The assay procedure was similar to the CPE assay, except for the end reading of the assay, which was performed on day 3 by measuring the relative fluorescence of treated
- 10 cultures and comparing this with the relative fluorescence of untreated cultures. The EC₅₀ or the EC₉₀ of a compound was defined as the concentration that inhibited the relative fluorescence by 50% or 90% respectively.

Antiviral assay with PBMC cultures

- The purification and activation of PBMCs as well as the antiviral assays were carried out
- 15 as described (CDER. Guidance for Industry Points to Consider in the Preclinical Development of Antiviral Drugs. 1990). The assay measured the extent that a drug inhibits HIV p24 antigen production by peripheral blood mononuclear cells (PBMC) cultures acutely infected with a viral strain. The susceptibility determination uses phytohaemagglutinine (PHA)-stimulated PBMCs from normal donors. In the *in vitro*
- 20 infection experiments 1000 CCID₅₀ per million PHA-stimulated PBMCs was used. Cultures were split 1/2 every 3 to 4 days and compound was added together with the addition of new medium.

- The p24 antigen production was measured using a commercial kit, according to the manufacturer protocol (NEN), at the moment that the p24 production of untreated
- 25 infected cultures is maximal; i.e. between 7 and 11 days after infection.

The % p24 production was calculated by means of following equation:

$$\%p24 = 100 \times \frac{[p24]_{\text{Sample}} - [p24]_{\text{Mock_Control}}}{[p24]_{\text{HIV_Control}} - [p24]_{\text{Mock_Control}}}$$

- where [p24]_{Sample} is the p24 concentration in an infected treated culture, [p24]_{HIV_Control} is the p24 concentration in an infected untreated culture and [p24]_{Mock_Control} is the p24
- 30 concentration in a mock-infected culture. The dose achieving 50% p24 production according to the above formula was defined as the EC₅₀, while the dose achieving 10% p24 production according to the above formula was defined as the EC₉₀.

Antiviral assay with monocytes/macrophages

The assay measured the extent that a drug inhibits HIV p24 antigen production by

primary monocytes/macrophages acutely infected with HIV-1/BaL (300 CCID₅₀/ml). The susceptibility determination used monocytes/macrophages isolated from PBMCs from normal donors by plastic adherence. Every 5 days cultures were fed with complete medium containing the appropriate compound concentrations. The p24 antigen
5 production was measured at day 14 after virus challenge and EC₅₀ and EC₉₀ values were calculated.

Recombinant virus assays

A recombinant virus assay (RVA) starts with the amplification of viral target sequences by means of PCR. The amplicons are incorporated into a proviral laboratory clone
10 deleted for the sequences, present in the amplicon. This generates a stock of recombinant viruses. The viruses are tested for their ability to grow in the presence of different concentrations of drugs. Results are obtained by calculating EC₅₀ values for each inhibitor and by reporting the results as EC₅₀ values, expressed in μ M concentrations, or by computing the ratio of the EC₅₀ values found for the recombinant
15 virus to the EC₅₀ values found for a wild type susceptible laboratory virus tested in parallel. In the latter case, resistance is expressed as "fold-resistance" (fold change in susceptibility, FC) compared to a wild-type susceptible HIV-1 strain.

The use of reporter gene systems for susceptibility testing allows the implementation of laboratory automation and standardization (Pauwels, et al., J.Virol. Methods 20, 309-
20 321 (1988); Paulous, S., et al., International Workshop on HIV Drug Resistance, Treatment Strategies and Eradication, St. Petersburg, Florida, USA. Abstr. 46 (1997); and Deeks, S. G., et al., 2nd International Workshop on HIV Drug Resistance and Treatment Strategies, Lake Maggiore, Italy. Abstr. 53 (1998)).

The Antivirogram® assay (Virco) (WO 97/27480) is based on homologous
25 recombination of patient derived HIV-1 *gag*/PR/RT sequences into a proviral HIV-1 clone correspondingly deleted for the *gag*/PR/RT sequences. A similar assay (Phenosense® ViroLogic, WO 97/27319) is based on enzymatic ligation of patient-derived PR/RT sequences into a correspondingly deleted proviral vector carrying an indicator gene, luciferase, inserted in the deleted HIV-1 envelope gene. Another assay
30 was developed by Bioalliance (Phenoscript, WO 02/38792). The development of high throughput phenotyping and genotyping assays has allowed the establishment of a database containing the phenotypic resistance data and the genotypic sequences of over 30,000 clinical isolates.

Experimental part

Example 1. The Identification of Mutational Patterns in HIV-1 Protease and the Correlated Phenotypic Resistance.

Plasma samples from HIV-1-infected individuals from routine clinical practice were obtained and shipped to the laboratory on dry ice and stored at -70°C until analysis. Viral RNA was extracted from 200 μL patient plasma using the QIAAMP[®] Viral RNA Extraction Kit (Qiagen, Hilden, Germany), according to the manufacturers instructions. cDNA encompassing part of the *pol* gene was produced using Expand[™] reverse transcriptase (Boehringer Mannheim). A 2.2kb fragment encoding the protease and RT regions were amplified from patient-derived viral RNA by nested polymerase chain reaction (PCR) using PCR primers and conditions as described. (Hertogs K., et al., Antimicrob. Agents Chemother. 42: 269-276 (1998), WO 01/81624). This genetic material was used in phenotyping and genotyping experiments.

Phenotypic analysis was performed using the recombinant virus assay (Antivirogram[®])(WO 97/27480). MT-4 cells (Harada S., et al, Science 229: 563-566 (1985).) were co-transfected with *pol* gene PCR fragments and the protease-RT deleted HIV-1 molecular clone, pGEM3 Δ PRT. This resulted in viable recombinant viruses containing protease/RT from the donor PCR fragment. After homologous recombination of amplicons into a PR-RT deleted proviral clone, the resulting recombinant viruses were harvested, titrated and used for *in vitro* susceptibility testing to antiretroviral drugs. The results of this analysis were expressed as fold change in susceptibility, reflecting the fold change in mean EC_{50} (μM) of a particular drug when tested with patient-derived recombinant virus isolates, relative to the mean EC_{50} (μM) of the same drug obtained when tested with a reference wild-type virus isolate (IIB/LAI). Genotyping was performed by an automated population-based full-sequence analysis, through a dideoxynucleotide-based approach, using the BigDye[™] terminator kit (Applied Biosystems, Inc.) and resolved on an ABI 377 DNA sequencer. The genotypes are reported as amino acid changes at positions along the protease gene compared to the wild-type (HXB2) reference sequence. Analysis by VirtualPhenotype[™] interpretational software (WO 01/79540) allowed detection of mutational patterns in the database containing the genetic sequences of the clinical isolates and linkage with the corresponding resistance profiles of the same isolates.

Example 2. Susceptibility analysis of HIV-1 variants constructed by site-directed mutagenesis

Mutations in the protease or RT coding region were created by site-directed mutagenesis, using the QuikChange[®] Site-Directed Mutagenesis Kit (STRATAGENE[®]), of a wild-type HXB2-D *EcoRI-PstI* restriction enzyme fragment, encompassing the HIV-1 *pol* gene and

cloned into pGEM3 (Promega). All mutant clones were verified by DNA sequence analysis. PCR fragments were prepared from the mutated clones and the altered protease coding regions were transferred into HIV-1 HXB2-D by homologous recombination as described above. The susceptibility of these recombinant viruses to drugs was determined by the MT-4 cell CPE protection assay.

Example 3. *In vitro* selection of resistant strains

MT4-LTR-EGFP cells were infected at a multiplicity of infection (MOI) of 0.01 to 0.001 CCID₅₀ / cell in the presence of inhibitor. The starting concentration of the inhibitor was two to three times the EC₅₀, a suboptimal concentration. The cultures were sub-cultivated and scored microscopically on virus-induced fluorescence and cytopathicity every 3 - 4 days. The cultures were sub-cultivated in the presence of the same compound concentration until signs of virus replication were observed. The escaping virus was further cultivated in the presence of the same inhibitor concentration in order to enrich the population in resistant variants. If full virus breakthrough was observed the supernatant was collected and stored (new virus strain). Afterwards, the same virus was challenged with a higher compound concentration in order to select variants able to grow in the presence of as high as possible inhibitor concentrations. From the new viruses, a virus stock was grown in the absence of inhibitor.

In vitro drug selection experiments starting from wild-type HIV-1 under pressure of compound 1, compound 2, and Nelfinavir (NFV) have been performed. Tables 1, 2, 3, 4, and 5 show the genotypic and phenotypic characterization of the selected strains.

Table 1 : Characterization of the strains isolated from HIV-1/LAI in the presence of compound 1

<i>In vitro</i> selection				
Experimental conditions				
Starting strain	HIV/LAI	HIV-1/LAI	HIV-1/LAI	HIV-1/LAI
Compound	-	Compound 1	Compound 1	Compound 1
Concentration (nM)	-	30	100	100
Days	-	45	97	188
Protease Genotype				
Mutations	-	R41T	R41T K70E	R41T K70E
Phenotype				
<i>In vitro</i> susceptibility to PIs				
N,median EC50 (nM),median FC				

Compound 1	N	37	7	6	3
	EC50	3.2	7.7	26	44
	FC	1	2	8	10
Indinavir	N	16	3	3	2
	EC50	28	33	98	140
	FC	1	1	4	5
Ritonavir	N	16	3	3	2
	EC50	31	32	21	46
	FC	1	1	1	1
Nelfinavir	N	11	3	4	2
	EC50	30	32	18	37
	FC	1	1	1	1
Saquinavir	N	46	2	6	3
	EC50	7.8	30	35	150
	FC	1	4	4	20
Amprenavir	N	67	3	6	3
	EC50	36	38	29	39
	FC	1	1	1	1
Lopinavir	N	11	3	5	3
	EC50	7.9	27	32	47
	FC	1	3	4	6

Table 2: Characterization of the strains isolated from HIV-1/LAI in the presence of compound 1

<i>In vitro</i> selection					
Experimental conditions					
Starting strain	HIV-1/ LAI	HIV-1/ LAI	HIV-1/ LAI	HIV-1/ LAI	HIV-1/ LAI
Compound	-	Comp 1	Comp 1	Comp 1	Comp 1
Concentration (nM)	-	30	100	100	200
Days	-	70	139	195	328
Protease Genotype					
Mutations	-		S37S/N R41R/K K70E	S37N R41S K70E	S37N R41S K70E
Phenotype					
<i>In vitro</i> susceptibility to PIs					
N, median EC₅₀ (nM), median FC					

Compound 1	N	5	1	1	1	1
	EC50	2.6	6.5	2.5	4.7	0.4
	FC	1	3	1	2	0.2
IDV	N	4	1	1	2	1
	EC50	12	18	6.3	9.1	5.5
	FC	1	2	1	1	0.5
RTV	N	3	1	1	2	1
	EC50	33	47	22	14	31
	FC	1	1	1	0.4	1
NFV	N	4	1	1	2	1
	EC50	38	39	9.7	9.5	1.9
	FC	1	1	0.3	0.3	0.1
SQV	N	3	1	1	1	1
	EC50	5.6	6.0	0.7	0.9	4.0
	FC	1	1	0.1	0.2	1
APV	N	5	1	1	2	1
	EC50	20	56	24	14	15
	FC	1	3	1	1	1
LPV	N	5	1	1	2	1
	EC50	4.6	17	2.8	3.9	1.1
	FC	1	4	1	1	0.2

Table 3: Characterization of the strains isolated from HIV-1/LAI in the presence of compound 2

<i>In vitro</i> selection				
Experimental conditions				
Starting strain	HIV-1/LAI	HIV-1/LAI	HIV-1/LAI	HIV-1/LAI
Compound	-	Compound 2	Compound 2	Compound 2
Concentration (nM)	-	100	100	100
Days	-	116	200	264
Protease Genotype				
Mutations	-	G16G/H	G16E	
		R41I	R41I	R41I
Phenotype				
<i>In vitro</i> susceptibility to PIs				
N, median EC ₅₀ (nM), median FC				
Compound 2	N	2	2	1
	EC50	12	6.9	61

	FC	1	1	5
IDV	N	4	1	1
	EC50	12	19	47
	FC	1	2	4
RTV	N	3	2	1
	EC50	33	22	23
	FC	1	1	1
NFV	N	4	2	1
	EC50	38	16	14
	FC	1	0	0
SQV	N	3		1
	EC50	5.6		45
	FC	1		8
APV	N	5	2	1
	EC50	20	14	8.4
	FC	1	1	0
LPV	N	5	2	1
	EC50	4.6	<0.9	18
	FC	1	0	4

Table 4: Characterization of the strains isolated from HIV-1/LAI in the presence of compound 1

<i>In vitro</i> selection						
Experimental conditions						
Starting strain	HIV-1/ LAI	HIV-1	HIV-1	HIV-1	HIV-1	
Compound	-	-	Comp 1	Comp 1	Comp 1	
Concentration (nM)	-	-	20	40	40	
Days	-	-	94	161	175	
Protease Genotype						
Mutations	-	-	R41G/R V82V/I	R41G V82I	R41G V82I	
Phenotype						
<i>In vitro</i> susceptibility to PIs						
N, median EC ₅₀ (nM), median FC						
Compound 1	N	5	1	1	1	1
	EC50	2.6	3.4	1.1	2.6	1.9
	FC	1	1	0	1	1
IDV	N	4	1	1	1	1
	EC50	12	2.4	3.1	2.1	3.9

	FC	1	0	0	0	0
RTV	N	3	1	1	1	1
	EC50	33	22	4.2	6.3	1.7
	FC	1	1	0	0	0
NFV	N	4	1	1	1	1
	EC50	38	31	5.7	11	16
	FC	1	1	0	0	0
SQV	N	3	1	1	1	1
	EC50	5.6	8.9	0.9	1.0	0.8
	FC	1	2	0	0	0
APV	N	5	1	1	1	1
	EC50	20	26	7.3	6.6	9.4
	FC	1	1	0	0	0
LPV	N	5	1	1	1	1
	EC50	4.6	6.8	2.0	1.8	1.0
	FC	1	1	0	0	0

Table 5: Characterization of the strains isolated from HIV-1/LAI in the presence of nelfinavir (NFV)

<i>In vitro</i> selection					
Experimental conditions					
Starting strain	HIV-1/ LAI	HIV-1/ LAI	HIV-1/ LAI	HIV-1/ LAI	HIV-1/ LAI
Compound	-	NFV	NFV	NFV	NFV
Concentration (nM)	-	1000	3000	9000	9000
Days	-	35	69	111	140
Protease Genotype					
Mutations	-		L10F	L10F	L10F
		D30N	D30N	D30N	D30N
				R41R/K	R41R/K
				K45I/K	K45I/K
		M46I	M46I	M46I	M46I
			V77I	V77I	V77I
				I84V/I	I84V
			I85V/I	I85V/I	
			N88D/N	N88D	N88D
Phenotype					
<i>In vitro</i> susceptibility to PIs					
N, median EC ₅₀ (nM), median FC					
IDV	N	4	1	1	1
	EC50	12	7.9	100	28

	FC	1	1		8	2
RTV	N	3	1	1	1	1
	EC50	33	19	27	86	170
	FC	1	1	1	3	5
NFV	N	4	1		1	1
	EC50	38	330		7200	6800
	FC	1	9		200	200
SQV	N	3	1	1	1	1
	EC50	5.6	1.8	2.5	15	34
	FC	1	0	0	3	6
APV	N	5	1	1	1	1
	EC50	20	28	59	95	190
	FC	1	1	3	5	10
LPV	N	5	1	1	1	1
	EC50	4.6	7.7	24	39	56
	FC	1	2	5	8	10

The *in vitro* antiviral activity of compound 1, compound 2, Nelfinavir, and current PIs against the selected strains was evaluated in acutely infected MT4 cells. Median EC₅₀ values together with the number of determinations (N), and the fold change in EC₅₀ as compared to wild type (FC) are reported.

Claims:

1. A computer system comprising at least one database correlating the presence of at least one mutation in a human immunodeficiency virus (HIV) protease and a change in susceptibility of at least one strain of HIV to a protease inhibitor, comprising at least one record corresponding to a correlation between at least one mutation selected from 41S, 41T, 41I, 41K, 41G and 70E in said protease, and treatment with at least a protease inhibitor.
2. A computer system comprising at least one database correlating the presence of at least one mutation in a human immunodeficiency virus (HIV) protease and a change in susceptibility of at least one strain of HIV to a protease inhibitor, comprising at least one record corresponding to a correlation between at least one mutation selected from 41T, 41I, 41K, 41G and 70E in said protease, and treatment with at least a protease inhibitor.
3. A method for evaluating the effectiveness of a protease inhibitor as an antiviral therapy for a patient infected with at least one mutant HIV strain comprising:
 - (i) collecting a sample from an HIV-infected patient;
 - (ii) determining whether the sample comprises a nucleic acid encoding HIV protease having at least one mutation selected from 41S, 41T, 41I, 41K, 41G and 70E;
 - (iii) correlating the presence of said at least one mutation of step (ii) to a change in effectiveness of said protease inhibitor.
4. A method for evaluating the effectiveness of a protease inhibitor as an antiviral therapy for a patient infected with at least one mutant HIV strain comprising:
 - (i) collecting a sample from an HIV-infected patient;
 - (ii) determining whether the sample comprises a nucleic acid encoding HIV protease having at least one mutation selected from 41T, 41I, 41K, 41G and 70E;
 - (iii) correlating the presence of said at least one mutation of step (ii) to a change in effectiveness of said protease inhibitor.
5. A method for identifying a drug effective against mutant HIV protease, comprising:
 - (i) providing a nucleic acid comprising mutant HIV protease comprising at least one mutation chosen from 41S, 41T, 41I, 41K, 41G and 70E;
 - (ii) recombining said nucleic acid comprising mutant HIV protease of step (i) into a proviral nucleic acid deleted for said sequence to generate a recombinant HIV

virus;

(iii) determining a phenotypic response to said drug for said HIV recombinant virus; and

(iv) identifying a drug effective against mutant HIV based on the phenotypic response of step (iii).

6. A method for identifying a drug effective against mutant HIV protease, comprising:

(i) providing a nucleic acid comprising mutant HIV protease comprising at least one mutation chosen from 41T, 41I, 41K, 41G and 70E;

(ii) recombining said nucleic acid comprising mutant HIV protease of step (i) into a proviral nucleic acid deleted for said sequence to generate a recombinant HIV virus;

(iii) determining a phenotypic response to said drug for said HIV recombinant virus; and

(iv) identifying a drug effective against mutant HIV based on the phenotypic response of step (iii).

7. A method for identifying a drug effective against mutant HIV protease, comprising:

(i) providing a HIV protease comprising at least one mutation chosen from 41S, 41T, 41I, 41K, 41G and 70E;

(ii) determining the activity of said drug on said HIV protease;

(iii) determining the activity of said drug on wild type HIV protease;

(iv) determining the ratio of the activity determined in step (iii) over the activity determined in step (ii);

(v) identifying an effective drug against mutant HIV based on the ratio of step (iv).

8. A method for identifying a drug effective against mutant HIV protease, comprising:

(i) providing a HIV protease comprising at least one mutation chosen from 41T, 41I, 41K, 41G and 70E;

(ii) determining the activity of said drug on said HIV protease;

(iii) determining the activity of said drug on wild type HIV protease;

(iv) determining the ratio of the activity determined in step (iii) over the activity determined in step (ii);

(v) identifying an effective drug against mutant HIV based on the ratio of step (iv).

9. A method for evaluating a change in viral drug susceptibility comprising:

- (i) collecting a sample from an HIV-infected patient;
- (ii) determining whether the sample comprises a HIV protease having at least one mutation selected from 41S, 41T, 41I, 41K, 41G and 70E;
- (iii) correlating the presence of said at least one mutation of step (ii) to a change in viral drug susceptibility.

10. A method for evaluating a change in viral drug susceptibility comprising:

- (i) collecting a sample from an HIV-infected patient;
- (ii) determining whether the sample comprises a HIV protease having at least one mutation selected from 41T, 41I, 41K, 41G and 70E;
- (iii) correlating the presence of said at least one mutation of step (ii) to a change in viral drug susceptibility.

11. A method for evaluating a change in viral drug susceptibility, comprising:

- (i) providing an HIV comprising a protease comprising at least one mutation chosen from 41S, 41T, 41I, 41K, 41G and 70E;
- (ii) determining a phenotypic response of said virus to said drug; and
- (iii) correlating the phenotypic response of step (ii) to a change in viral drug susceptibility.

12. A method for evaluating a change in viral drug susceptibility, comprising:

- (i) providing an HIV comprising a protease comprising at least one mutation chosen from 41T, 41I, 41K, 41G and 70E;
- (ii) determining a phenotypic response of said virus to said drug; and
- (iv) correlating the phenotypic response of step (ii) to a change in viral drug susceptibility.

13. A method for identifying a drug effective against mutant HIV protease, comprising:

- (i) providing a HIV protease comprising at least one mutation chosen from 41S, 41T, 41I, 41K, 41G and 70E;
- (ii) determining the activity of said drug towards said protease;
- (iii) determining the activity of said drug to wild type HIV protease and;
- (iv) determining the ratio of the activity determined in step (iii) over the activity determined in step (ii);
- (v) identifying an effective drug against mutant HIV based on the ratio of step (iv).

14. A method for identifying a drug effective against mutant HIV protease, comprising:

- (i) providing a HIV protease comprising at least one mutation chosen from 41T, 41I, 41K, 41G and 70E;

- (ii) determining the activity of said drug towards said protease;
- (iii) determining the activity of said drug to wild type HIV protease and;
- (iv) determining the ratio of the activity determined in step (iii) over the activity determined in step (ii);
- 5 (v) identifying an effective drug against mutant HIV based on the ratio of step (iv).

15. A vector for performing phenotypic analysis comprising an HIV sequence having at least one mutation in the HIV protease gene chosen from 41S, 41T, 41I, 41K, 41G and 70E.

10

16. A vector for performing phenotypic analysis comprising an HIV sequence having at least one mutation in the HIV protease gene chosen from 41T, 41I, 41K, 41G and 70E.

15 17. An isolated and purified HIV protease sequence having at least one mutation selected from 41S, 41T, 41I, 41K, 41G and 70E, wherein said at least one mutation in said sequence correlates to a fold change in susceptibility towards a HIV protease inhibitor.

20 18. An isolated and purified HIV protease sequence having at least one mutation selected from 41T, 41I, 41K, 41G and 70E, wherein said at least one mutation in said sequence correlates to a fold change in susceptibility towards a HIV protease inhibitor.

25 19. An isolated and purified oligonucleotide comprising a HIV protease sequence of 5 to 100 bases for in vitro diagnosis of viral drug resistance, characterized in that said oligonucleotide comprises at least one mutation chosen from 41S, 41T, 41I, 41K, 41G and 70E.

30 20. An isolated and purified oligonucleotide comprising a HIV protease sequence of 5 to 100 bases for in vitro diagnosis of viral drug resistance, characterized in that said oligonucleotide comprises at least one mutation chosen from 41T, 41I, 41K, 41G and 70E.

35